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CHAPTER 11

An Artificial Microbial Ecosystem for Determining Effects and Fate of Toxicants in a Salt-Marsh Environment*

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An artificial laboratory environment designed to determine microbial interactions with pollutant chemicals is proposed. The system is designed to obtain maximum reproducibility between replicates by dividing a single tank into separate closed chambers. Radiolabeled toxicants are added directly to the core-chambers and monitored for metabolic breakdown. Further information is obtained easily on changes in microbial, physiological indexes induced by the toxicants. Techniques for monitoring effects of the methyl parathion on the microbial population and the fate of this chemical are given.

INTRODUCTION

The microbial communities of salt-marsh environments along the Gulf and Atlantic coasts have been extensively studied for the past 20 years (Burkholder and Bornside 1957; Day et al. 1973; Fenchel 1969). However, much remains to be learned concerning interrelationships of the diverse microbial communities and their role in carbon metabolism within the estuarine environment. Previous studies have been concerned primarily with numerical and taxonomic aspects of the heterotrophic communities. Varied populations of bacteria (*Bacillus, Serratia, Flavobacterium, Achromobacter, Pseudomonas*), yeast, and fungi (Meyers 1971) and many protozoan and micrometazoan species (Lackey 1967) are isolated commonly from decaying salt-marsh grasses. Recently, several studies have shown the importance of the various microbial communities in mineralization or uptake of nutrients that result from primary production in vast stands of salt-marsh grasses, i.e., *Spartina* sp. or *Juncus* sp. (Fallon and Pfaender 1976; Gallagher et al. 1975; Gosselink and Kirby 1974). Using antibiotics to inhibit selectively portions of the microbial community, Fallon and Pfaender (1976) demonstrated that the heterotrophic bacteria were the most important component responsible for the mineralization process.

Persistent chemical pollutants, such as pesticides, eventually enter estuaries as a result of agricultural runoff, industrial effluents, and the direct application in or near salt-marshes (Butler 1973). Concern for possible hazards to nontarget species, including disruption of the heterotrophic decomposition process in the salt marsh, has prompted studies to determine the fate and effects of these chemical toxicants. Laboratory studies on the microbial metabolism of these toxicants, while contributing significantly to the knowledge of biochemical pathways for single species under ideal conditions, do not always describe metabolic fate under more

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"natural" conditions. Therefore, our laboratory attempted to design a realistic method for screening new pesticides for environmental toxicity and metabolic fate in a multi-species, artificial estuarine environment. As part of this approach, we have developed an artificial salt-marsh environment for studying (1) the effects of these toxicants on the heterotrophic community of the salt marsh, and (2) the fate of the pollutant due to microbial metabolism. This paper presents the design and methodology of an artificial salt-marsh microcosm used to obtain information on pesticide processes and effects; methyl parathion was used as a reference toxicant to demonstrate the type of information obtained using this system.

Materials and Methods

Preparation and use of artificial laboratory environments (core-chamber method). Water and sediment (sand) were collected from a pristine salt marsh on Santa Rosa Island, near Pensacola Beach, Florida. Sediments were sieved through a nylon screen (2 mm^2 mesh) and washed on site with salt-marsh water to remove large particulates. Sieved sand and water were transported to the laboratory (within 15 min) and placed in an acetone-cleaned 10-gal glass aquarium. The salt-marsh sediment was approx. 6 cm deep and the water column was 20 cm deep (30 liters). The artificial environment was covered with a plexiglass template that permitted later insertion of core-chambers (microcosms), aerated, and allowed to acclimate at a constant temperature (28 C) for 24 h. Then 15 sterile glass cylinders (3.5 X 30 cm) were inserted through the water column into the sediments and firmly affixed to silicone stoppers (No. 7) placed below the sediments before adaptation (Fig. 1). Each chamber contained 200 ml of marsh water and 30 cm^3 of sediment, and was fitted with its own continuous aeration system using air which was passed through a column (4 X 20 cm) of Ascarite[1] (A. H. Thomas Co., Phila., PA) to remove the CO_2; the air entered a manifold that diverted the flow into each microcosm. Air-flow rates into each chamber were regulated by needle valves. Exhaust gases were passed through resin traps (0.5 X 10 cm, Amberlite XAD-4, Rohm and Haas, Phila., PA) to remove all volatilized organics, and then through tubes that contained 15 ml of 1.0 M KOH to trap CO_2.

Methyl parathion was added by pipette to each chamber at concn of 0.1, 1.0, and 10 mg/liter, each containing 22 μCi ^{14}C in 1.0 ml acetone. Three replications per concn were used. Controls consisted of three unaltered chambers, three chambers with carrier alone, and three sterile (autoclaved) chambers to which the toxicant was added.

Samples of the water column were taken from each cylinder by temporarily removing the exhaust line and inserting a thin length of sterile teflon tubing affixed to a sterile, graduated syringe. Samples were taken 0, 3, 6, 8, 10, 12, 14, 16, 18, and 20 days after injection of toxicant. One milliliter was added directly to a sterile 9-ml seawater blank for serial dilutions for bacterial enumeration and 5 ml were taken for radioassay and chemical analyses.

Experimental procedures. Total aerobic bacteria were enumerated by the standard plate-count method on ZoBell's marine agar (15%), while amylolytic, proteolytic (casein and gelatin), and phospholytic bacteria were enumerated as described by Colwell and Wiebe (1970). Chitinolytic bacteria were enumerated as described by Hood and Meyers (1974) and

[1] Mention of commercial products or trade name does not constitute endorsement by U.S. Environmental Protection Agency.

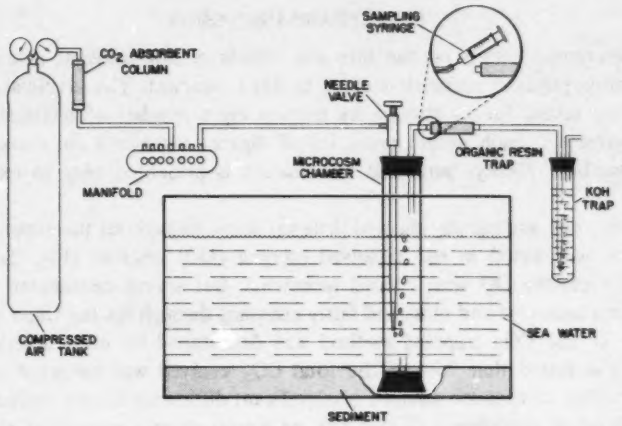

FIG. 1. Apparatus for core-chamber system. Sediment and water are obtained directly from the salt-marsh environment and "cored" by sterile glass cylinders after adaptation to the laboratory environment. CO_2-free air is used to aerate each of 15 separate core-chambers; total CO_2 is collected from the exit tubes. Volatile organics are collected on XAD resin in the exit tube. The core-chambers are sampled through the exit tube.

cellulolytic bacteria were determined by the MPN method as described by Rodina (1972).

Dissolved oxygen was determined at various depths of the water column using a dissolved oxygen meter (New Brunswick Scientific Co., New Brunswick, NJ), calibrated to 100% in air-saturated water. The pH was determined with a Corning pH meter; salinity was measured with a salinometer/refractometer (American Optical Corp., Keene, NH). Carbon dioxide was measured by absorption in KOH traps. The traps were removed at 48-h intervals and excess $BaCl_2$ (2.5 N) was added to precipitate the CO_2 as $BaCO_3$. The precipitate was collected on a membrane filter (0.45 μm), dried at 60 C for 24 h, and weighed. Total CO_2 evolved was calculated in millimoles of CO_2. The entire $BaCO_3$ sample was placed into a scintillation cocktail and assayed for radioactivity.

Water samples for radioassay were extracted with two volumes of methylene chloride; the water was acidified to pH 2 with concentrated H_2SO_4 and re-extracted with two volumes of methylene chloride; the extracts were pooled. One milliliter of both solvent-extractable and nonextractable fractions was placed in a scintillation cocktail (toluene:ethylene glycol monomethyl ether, 2:1 v/v, plus 0.55% 2,5 diphenlyoxazole) and assayed for radioactivity with a Beckman Model LS-250 spectrophotometer (Beckman Instrument Inc., Fullerton, CA). The solvent-extractables were separated by thin-layer chromatography (TLC) on prewashed Silica gel F-254 glass plates (Brinkman Inc., Westbury, NY), using an appropriate solvent system (diethyl ether:hexane, 75:25). Radioactive compounds were located using an autoradiograph spark chamber (Birchover Instruments, Ltd., Letchworth, Herts, England) and identified by comparison with known standards. Quantitation of compounds was made by scraping the spots from the TLC plates and assaying for radioactivity.

XAD-resin traps were removed at 14 and 21 days and eluted with acetonitrile and assayed for radioactivity. At the end of the experiment, the core-chambers were rinsed twice with methylene chloride. The washings and the remaining solvent extractables from the water column were assayed for radioactivity.

Results and Discussion

To obtain meaningful results on the fate and effects of any toxicant in an artificial environment, various physical parameters must be held constant. The artificial ecosystem presented here was tested for its stabilty by monitoring a number of physical, chemical, and biological parameters. Each datum point for all figures represents the average of values for three core-chambers. Methyl parathion information is presented only to exemplify typical results.

Temperature, pH, and salinity showed little variation throughout the experiment; however, some variation was noted in the dissolved oxygen (DO) content (Fig. 2). At the water-sediment interface, the DO was reduced somewhat, but several centimeters above the sediment, DO concn increased and remained fairly constant throughout the water column.

Efficiency of the CO_2 trapping method was determined by using several CO_2 traps in sequence. It was found that 75% of the total CO_2 evolved was collected in the first trap (Table 1). Analysis of variance revealed no significant difference among replica core-chambers at the 95% level of confidence ($F = 1.12$), or among sample periods at the 95% level of confidence ($F = 0.52$).

Heterotrophic biomass and heterotrophic CO_2 show an overall correlation (Fig. 3). Similarities can be seen in the curves for control (A), acetone control (B), and the toxicant-treated (C) core-chambers. However, within the toxicant-treated chambers, an early inhibitory response (1 day) in total CO_2 evolved was not reflected in total biomass. This lack of response, in all likelihood, was due to changes in selected physiological groups of bacteria which rapidly acclimated to the pertubations within the core-chamber. Total CO_2 appeared to be a more sensitive measure of inhibitions than microbial numbers.

Microbial metabolism of a toxicant can be followed with time and quantitated by using radiolabeled toxicants. TLC-autoradiographs of organic solvent-extractable metabolites of methyl parathion are shown in Fig. 4. The parent compound traveled farthest in this solvent system and appeared as the uppermost spot. This spot decreased in intensity with time. The

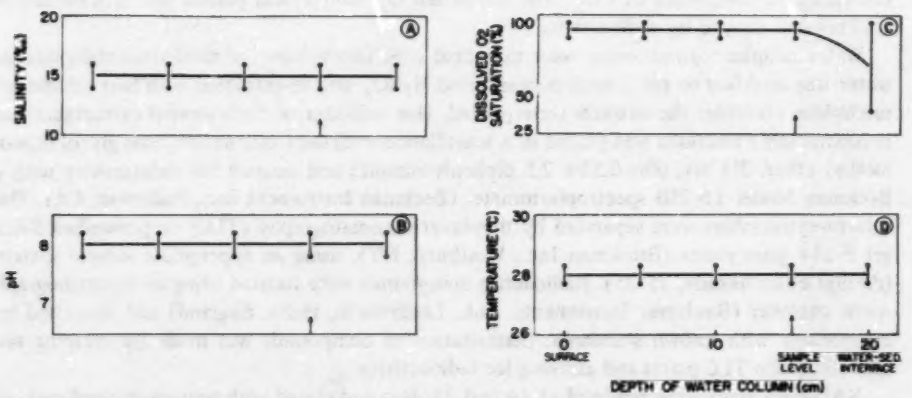

FIG. 2. Stability of physical parameters within core-chambers. Arrows denote level of sampling within core. A = salinity; B = pH; C - dissolved oxygen; D = temperature. Each point represents the average for three core-chambers.

TABLE 1. *Efficiency of CO_2 trapping method*

Replicate Chambers	Sample Periods (48 h each)		
	I	II	III
C_1	71[a]	68	67
C_2	81	69	70
C_3	70	–	65
C_4	71	76	62
C_5	74	–	74
C_6	72	68	76
C_7	72	64	86
C_8	71	62	77
C_9	70	71	71
C_{10}	–	73	51
C_{11}	76	62	61
C_{12}	74	70	75
C_{13}	68	70	66
C_{14}	72	69	77
C_{15}	76	–	81

[a]Percentage of total CO_2 evolved collected in a single trap.
Variation among replica chambers, F = 1.12
Variation among sample periods, F = 0.52

appearance of metabolites after 8 days was indicated by the slower moving spots. The continued intensity of these spots indicated increased concn of metabolites. The metabolites were removed from the TLC-plate, and quantitated to determine the extent of transformation of the compound. After exposure in the system for 12 days an intense spot of radioactivity remained at the origin with less parent compound detected. The responsible compounds at the origin could represent more polar metabolites. Further metabolism resulted in less extractable radioactivity after 14 days; increased levels of $^{14}CO_2$ detected in the gas traps indicated increased metabolism of these compounds. Comparisons with the sterile chambers indicated that microbial populations in the core-chambers had adapted to complete the breakdown of the toxicant as can be seen from the metabolites detected.

One problem associated with laboratory ecosystem studies is designing a system that can produce statistically reliable results. When mixed microorganisms and substrates from natural systems are adapted to the laboratory environment in individual tanks or bottles, each system can be different from its alleged duplicate. The core-chamber design of the single, adapted laboratory tank gives reliable, reproducible data among replicates. Another advantage of the core-chamber system is its compactness. The entire system, with its multiple core-chambers, can be set-up in one 10-gal aquarium that requires much less incubator space than separate multiple chambers of larger design. To compare a laboratory-adapted system with a natural ecosystem, unadapted environmental cores can be inserted into the core-chamber aquarium without disturbing the original laboratory system. However, environmental samples lead to greater variability among replicates than the homogeneous cores described here (Bourquin 1975). Our laboratories are currently developing the system for *in situ* use. Since variation among chambers is less than in other systems (Bourquin 1975), information generated with

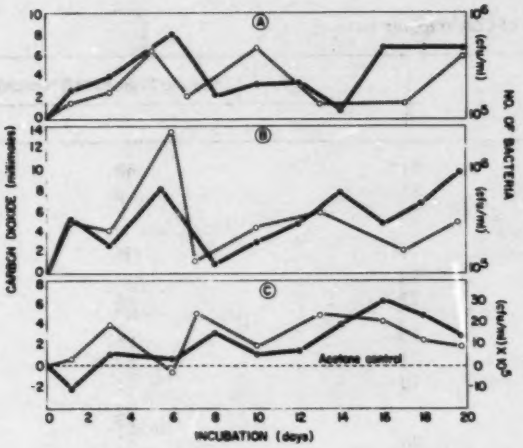

FIG. 3. Correlation of heterotrophic activity (bacterial concn) with total CO_2 evolution. (○) No. of bacteria; (●) Millimoles carbon dioxide; A = Unaltered control core-chambers; B = Acetone-treated control core-chambers; C = Experimental core-chamber; treated with 10 mg/liter of methyl parathion; data have been normalized to the acetone control, 3-B. Each point represents the average for three core-chambers.

this system should be more reliable statistically. We believe that our system can be used to study fate and effects of xenobiotics in estuarine and other aquatic environments. Investigations in our laboratory have studied successfully ^{14}C-pesticides in salt-marsh ecosystems. The fate and effects of both persistent and biodegradable pesticides in this artificial ecosystem are currently under study.

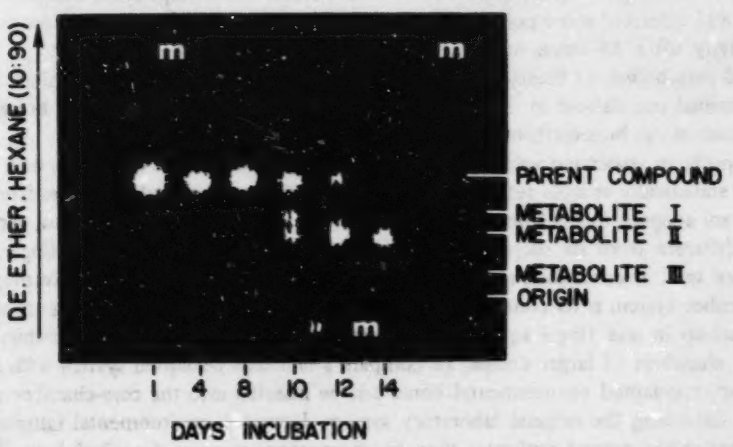

FIG. 4. Autoradiograph of thin-layer chromatogram of methyl parathion and its metabolites incubated in the core-chamber ecosystem. M = orientation mark.

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